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Phospholipase D2 downregulation induces cellular senescence through a reactive oxygen species–p53–p21$^{Cip1/WAF1}$ pathway

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ABSTRACT

The expression of phospholipase D1 (PLD1) and PLD2 were found to decrease at the transcription level during both replicative and premature senescence in human lung fibroblast IMR-90 cells. Knockdown of PLD2 dramatically induced senescent phenotype in proliferating IMR-90 cells and wild-type HCT116 colon cancer cells, whereas this response was nearly abolished in p53- or p21$^{Cip1/WAF1}$-null HCT116 cells. PLD2 knockdown increased the intracellular reactive oxygen species (ROS). Antioxidant N-acetyl-L-cysteine, NADPH oxidase inhibitor apocynin, and p22^{phox} small interfering RNA (siRNA) reduced ROS generation and thus suppressed the appearance of senescence markers. Elevated CK2 α subunit (CK2α) expression repressed PLD2 downregulation-mediated senescence. PLD2 overexpression increased protein kinase CK2 (also known as casein kinase 2) (CK2) activity. Taken together, these results show that PLD2 downregulation causes senescence through the p53–p21$^{Cip1/WAF1}$ pathway by stimulating ROS production, which is induced by CK2 inhibition.

1. Introduction

Phospholipase D (PLD) hydrolyzes phosphatidylcholine to generate the lipidic second messenger, phosphatidic acid (PA) and free choline. Numerous second messengers have been implicated in a wide range of biological functions including membrane vesicle trafficking, exocytosis, cell migration, anti-apoptotic signaling, cytoskeletal reorganization, and mitogenesis [1,2]. Two isoforms of mammalian PLD, namely, PLD1 and PLD2, have been reported. They share about 50% amino acid similarity, but exhibit different regulatory properties. PLD1 (120 kDa) has a low basal activity and is activated by phosphatidylinositol 4,5-bisphosphate (PIP2), protein kinase C (PKC), and members of the RhoA and ADP-ribosylation factor (ARF) families of small G proteins. In contrast, PLD2 (106 kDa) exhibits a higher basal level of cellular activity than PLD1, and is less responsive to PKC, RhoA, and ARF. PLD1 and PLD2 show a diverse subcellular localization. PLD activity is known to increase in response to mitogenic signals and activated oncoproteins such as EGF, v-Src, and v-Ras. PLD is also implicated in cell proliferation and cancer with an upregulation of its activity and expression in several types of human cancers [3,4]. Various chromosomal alterations in the PLD gene loci have been found in the ovarian, lung, breast, esophageal, and prostate cancers [5–7].

Normal primary cells enter an irreversible state of growth arrest termed "replicative senescence" after a finite number of cell divisions [8]. Replicative senescence is primarily driven by telomere shortening, which is recognized as DNA damage in cells. On the other hand, various forms of stress such as reactive oxygen species (ROS) and oncogenic activation induce an acute form of senescence called premature senescence [9–11]. Two tumor suppressor genes, p53 and Rb, have been implicated in the induction of replicative and premature senescence, called collectively as cellular senescence. Cellular senescence is also characterized by molecular and phenotypic characteristics, including the appearance of senescence-associated β-galactosidase activity (SA-β-gal) and the accumulation of p21$^{Cip1/WAF1}$ [12,13].

It has been shown that a decrease in the PLD activity may be associated with senescence. The senescence induction with ceramide may mediate PLD inactivation by exerting an inhibitory effect on PLD activators including PKC and RhoA [14–16]. However, the

Abbreviations: CK2, protein kinase CK2 (also known as casein kinase 2); CK2α, CK2 α subunit; DCF, dichlorofluorescein; DHE, dihydroethidium; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; FBS, fetal bovine serum; HA, hemagglutinin; NAC, N-acetyl-L-cysteine; NOX, NADPH oxidase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PDL, population doubling levels; PKC, protein kinase C; PLD, phospholipase D; ROS, reactive oxygen species; SA-β-gal, senescence-associated β-galactosidase; SDS, sodium dodecyl sulfate; siRNA, small interfering RNA

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precise nature of the mechanisms underlying the initiation and progression of PLD-mediated senescence remains unknown. In the present study, we investigated the physiological significance of PLD2 in the context of cellular senescence. Our results indicate that not only decreased PLD2 expression at the transcriptional level parallels with replicative and premature senescence in human fibroblasts, but also the downregulation of PLD2 induces senescence via a ROS–p53–$p21^{Cip1/WAF1}$ pathway.

2. Materials and methods

2.1. Materials

Antibodies against PLD, CK2α, CK2β, p53, $p21^{Cip1/WAF1}$, $p22^{phox}$, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-hemagglutinin (HA) antibody was obtained from Roche (Basel, Switzerland). Rhodamine-conjugated goat anti-rabbit IgG, CM-H_2DCFDA, and dihydroethidium (DHE) were purchased from Invitrogen (Carlsbad, CA). CK2 peptide substrate (RRREEETEEE) was synthesized using an automatic synthesizer (Model 431A, Applied Biosystems) and purified by reverse phase chromatography. [γ-^{32}P]ATP was purchased from Amersham Pharmacia Biotech (Korea).

2.2. Cell culture

Human diploid fibroblast IMR-90 cells were obtained from ATCC (Manassas, VA) at a population doubling level (PDL) of 24. IMR-90 cells and HCT116 human colon cancer cells (wild-type, $p53^{-/-}$, and $p21^{-/-}$) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) under a humidified atmosphere of 5% (v/v) CO_2 at 37 °C. The number of population doublings (PD) was calculated as PD = log (N_f/N_i)/log2, where N_f is the final cell number and N_i is the initial number of seeded cells. For premature senescence, the cells were exposed to multiple concentrations of H_2O_2 diluted in DMEM containing 10% FBS, for 2 h. The cells were washed with ice-cold phosphate-buffered saline (PBS) and then provided with fresh medium for 4 days prior to harvest or staining.

2.3. RNA interference and DNA transfections

The PLD2 siRNA, 5′-AAGAGGUGGCUGGUGGUGAAGdTdT-3′; CK2 siRNA, 5′-UCAAGAUGACUACCAGCUGdTdT-3′ and the negative control siRNA, 5′-GCUCAGAUCAAUACGGAGAdTdT-3′ were used. The siRNA for $p22^{phox}$ was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Cells were transfected with siRNAs using Lipofectamine (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Five hours after transfection, the medium was replaced with fresh medium, and the cells were grown for 2 days before being harvested. To induce overexpression of PLD2 and CK2α, cells were transfected with pcDNA-PLD2 or pcDNA-CK2α by Lipofectamine, according to the manufacturer's instructions.

2.4. Preparation of cell extracts and Western blotting

For the preparation of cell extracts, cells were lysed by sonication in lysis buffer [50 mM Tris–HCl (pH 8.0), 20 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 4 mM p-nitrophenyl phosphate, and protease inhibitor mix]. The particulate debris was removed by centrifugation at 12000×*g*, the volume of the supernatants was adjusted for equal protein concentration, and Western blotting was performed as described previously [17].

2.5. Reverse transcription-PCR (RT-PCR)

Total RNA was extracted from HCT116 cells. RNA was reverse-transcribed using gene-specific reverse primers and Reverse Transcriptase (Takara, Japan), and the resulting cDNA was PCR-amplified. The primer sequences used for PCR include: PLD1, PLD1-Fwd (5′-TGCTCTACAGGCAATCATGC-3′) and PLD1-Rev (5′-GCACTGTAGCCGAAGTCCTC-3′); PLD2, PLD2-Fwd (5′-CAGCAGGGACTCTGGAGAAC-3′) and PLD2-Rev (5′-TGAATACTCCCCACGACACA-3′). Primers for β-actin were used to standardize the concentration of RNA in each sample. PCR products were resolved on a 1.5% agarose gel, and quantification of the RT-PCR bands was performed using densitometry.

2.6. Immunocytochemical staining

Cells were seeded on to 4-well micro-chamber slides (Nunc, Naperville, IL) and fixed using 4% paraformaldehyde in PBS for 10 min at room temperature and then permeabilized in 0.25% Triton X-100 before blocking with 2% bovine serum albumin in PBS. The cells were incubated with primary antibodies (anti-PLD2, 1:200) at room temperature for 1 h, followed by incubation with the secondary rhodamine-conjugated goat anti-rabbit IgG (1:200) antibody. 4′,6-Diamidino-2-phenylindole (DAPI) (Molecular Probes, Eugene, OR) was used to counterstain the nuclei, and fluorescence signals were detected using a Carl Zeiss Axioplan 2 Microscope.

2.7. Colorimetric PLD activity assay

PLD activity was measured using a PLD activity colorimetric assay kit (BioVision, Inc., Milpitas, CA) according to the manufacturer's instructions. In this assay, PLD cleaves choline from phosphatidylcholine. The free choline is then oxidized to produce an intermediate that reacts with PLD probes to generate color (OD 570 nm).

2.8. CK2 activity assay

The standard assay for phosphotransferase activity of CK2 was conducted in a 30 μL reaction mixture containing 20 mM Tris–HCl (pH 7.5), 120 mM KCl, 10 mM $MgCl_2$, and 100 μM [γ-^{32}P]ATP in the presence of 1 mM synthetic peptide substrate (RRREEETEEE) at 30 °C. Cell lysates were added to initiate the reaction and incubated for 15 min. The reaction was stopped by the addition of trichloroacetic acid to a final concentration of 10%. The mixture was centrifuged, and then 10 μL of the supernatant was applied to P-81 paper. The paper was washed in 100 mM phosphoric acid, and radioactivity was measured by scintillation counting.

2.9. Measurement of intracellular reactive oxygen species (ROS) and SA-β-gal activity

Intracellular ROS and SA-β-gal activity were measured as described previously [17].

2.10. Statistical analysis

The results are presented as mean ± standard error. Statistical analysis was performed using SPSS version 11.0 (SPSS Inc., Chicago, IL). Data were analyzed by one-way ANOVA, and Duncan's multiple-range test was performed if differences were identified among groups at P of <0.05. Statistical difference between two groups was determined by unpaired Student t-test. A P value of <0.05 was considered to be statistically significant.

3. Results

3.1. Reduced expression of PLD1 and PLD2 during replicative and premature senescence in IMR-90 cells

To determine how the expression patterns of PLD1 and PLD2 are affected by replicative senescence, we repeatedly passaged IMR-90 cells until a senescence-like state was observed, as evidenced by a flattened cell morphology and SA-β-gal staining. Western blot analysis revealed that the expression of PLD1 and PLD2 in senescent cells (PDL 53) was significantly reduced in comparison to that in the proliferating cells (PDL 30) (Fig. 1A). In contrast, the level of p53 and $p21^{Cip1/WAF1}$ proteins increased in the senescent cells. We then examined whether this decrease in the amount of PLD proteins correlated with a decrease at the transcriptional level. RT-PCR results indicated that the mRNA expression of PLD1 and PLD2 significantly decreased in replicatively senescent cells (Fig. 1B). Quantification of PLD1 and PLD2 protein and mRNA levels showed that they were reduced by approximately 60% in senescent cells compared to proliferating cells (Fig. 1A and B). We next examined if PLD1 and PLD2 expression decreased in H_2O_2-induced senescent IMR-90 cells. Four days after exposure to 100–400 μM H_2O_2, proliferating IMR-90 cells (PDL 32) exhibited a senescent phenotype in a dose-dependent manner with a concomitant decrease in the protein levels of PLD1 and PLD2 (Fig. 1C). The mRNA expression of PLD1 and PLD2 also decreased in a dose-dependent manner in H_2O_2-treated cells (Fig. 1D). Since PLD2 has a high basal activity in various cells, the present study focused on the function of PLD2 in senescence. We investigated the intracellular localization of PLD2 in proliferating and senescent IMR-90 cells using immunocytochemical analysis. DAPI was used to counterstain the nuclei. While significant PLD2 expression was detected in the cytoplasm and the nucleus of proliferating IMR-90 cells, PLD2 expression was markedly reduced in senescent IMR-90 cells. The subcellular distribution of PLD2 remained essentially unchanged during senescence (Fig. 1E). We examined whether PLD catalytic activity was modulated during replicative senescence. PLD activity decreased by 25% in senescent cells compared to proliferating cells (Fig. 1F). Taken together, these results indicate that PLD is downregulated in IMR-90 cells during replicative as well as premature senescence.

3.2. Downregulation of PLD2 induces senescence through a $p53–p21^{Cip1/WAF1}$ pathway

To investigate the role of PLD2 in cellular senescence, we downregulated PLD2 using siRNA in proliferating IMR-90 cells (PDL 32) and found an increased rate of SA-β-gal staining in proliferating cells, compared with the control cells (Fig. 2A). Similarly, the accumulation of p53 and $p21^{Cip1/WAF1}$ proteins was observed in cells treated with PLD2 siRNA, in comparison with the controls (Fig. 2B). To examine the role of p53 and $p21^{Cip1/WAF1}$ in senescence induced by PLD2 downregulation, we used sublines of the human colon cancer cell line, HCT116, in which the *p53* or $p21^{Cip1/WAF1}$ genes are inactivated by homozygous knockout [12,18,19]. In the wild-type HCT116 cells, significant increase in SA-β-gal activity in response to PLD2 knockdown was observed. However, p53- and $p21^{Cip1/WAF1}$-negative cells did not show senescence, demonstrating that p53 and $p21^{Cip1/WAF1}$ are required for the PLD2 knockdown-mediated induction of senescence (Fig. 2C). When the protein levels of p53 and $p21^{Cip1/WAF1}$ were determined by Western blot, the expression of p53 was found to be upregulated in both the wild type and $p21^{Cip1/WAF1-/-}$ cells treated with PLD2 siRNA, but not in $p53^{-/-}$ cells. The expression of $p21^{Cip1/}$

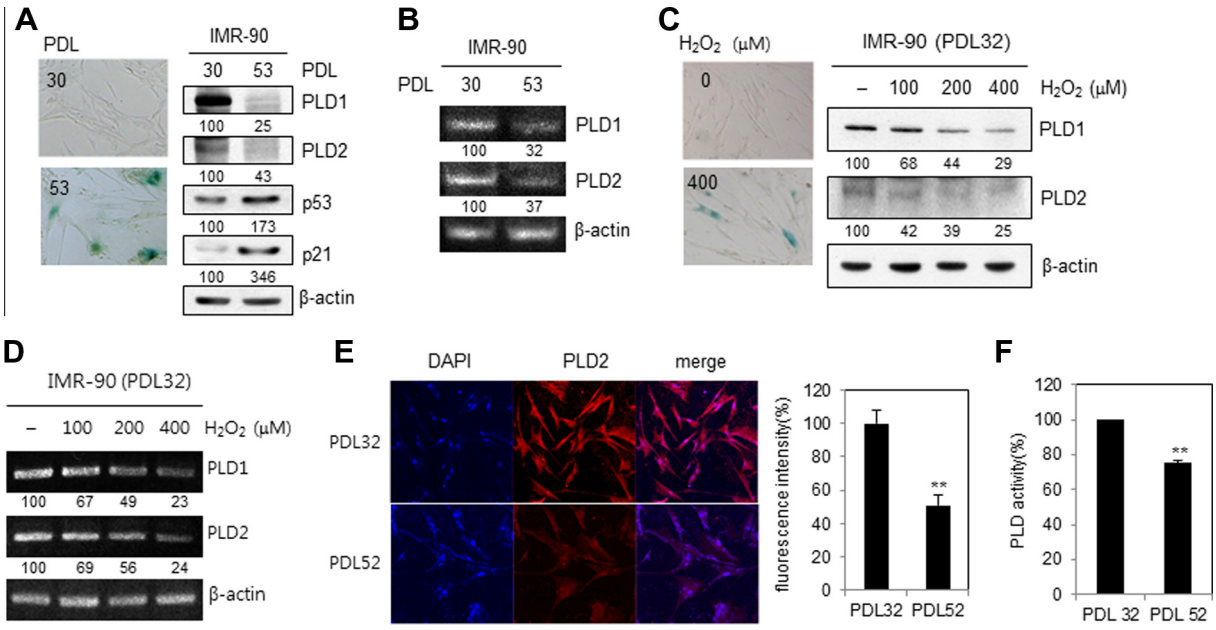

Fig. 1. Decrease in the expression of PLD1 and PLD2 during senescence in IMR-90 cells. (A) After serial passaging, IMR-90 cells at PDL of 30 and 53 were obtained. Cells were fixed and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside as described in Section 2. Representative images were obtained at 20× magnification (left panel). Cells were lysed, subjected to electrophoresis on a 10% (w/v) SDS–polyacrylamide gel, and visualized by Western blotting with the appropriate antibodies (right panel). β-actin served as the loading control. (B) Total RNA was extracted from cells and reverse-transcribed using PLD1- or PLD2-specific primers and reverse transcriptase. PCR products were resolved on a 1.5% (w/v) agarose gel. (C) IMR-90 cells (PDL 32) were treated with multiple concentrations of H_2O_2 at 37 C for 2 h. After removing H_2O_2 by changing the medium, the cells were incubated in fresh culture medium for 4 days and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside (left panel). Cells were lysed, subjected to electrophoresis on a 10% (w/v) SDS–polyacrylamide gel, and visualized by Western blotting with the appropriate antibodies (right panel). (D) Total RNA was extracted from H_2O_2-treated cells and reverse-transcribed using PLD1- and PLD2-specific primers and reverse transcriptase. Values below each band represent the mean fold differences ($n = 3$) in expression levels compared to the PDL 30 (A and B) or H_2O_2 minus control (C and D), which were assigned a value of 100. (E) IMR-90 cells were seeded onto Nunc chamber slides and the subcellular distribution of PLD2 (rhodamine) was assessed by immunocytochemical staining. Cells were counterstained with DAPI to visualize the nuclei (blue). (F) IMR-90 cells at PDL 32 and 53 were collected, and PLD activity was determined as described in the Section 2.

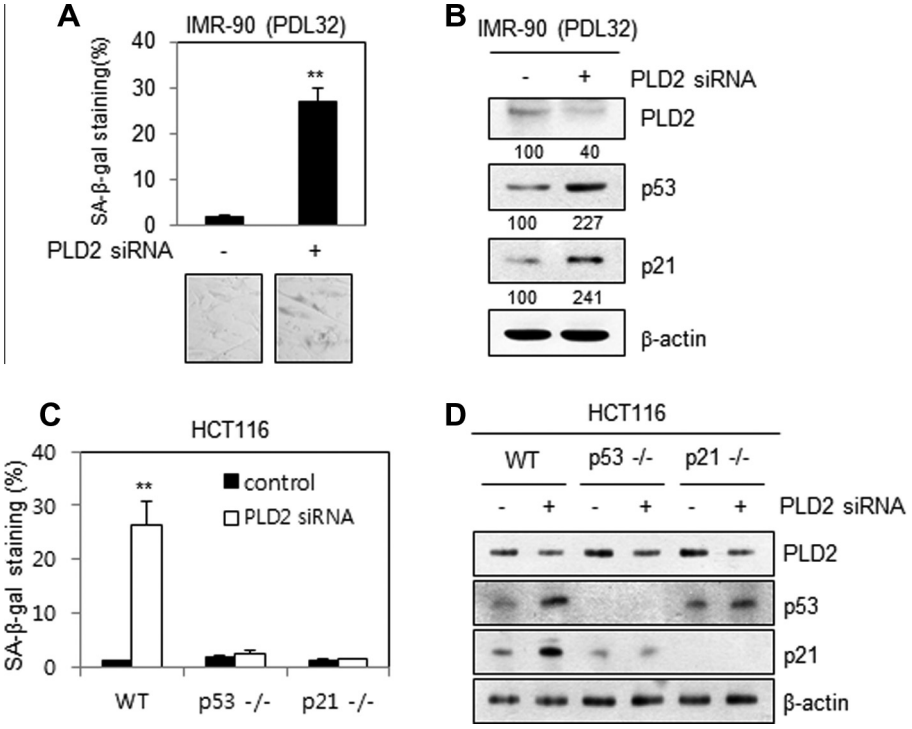

Fig. 2. Downregulation of PLD2 induces premature senescence through a p53–p21$^{Cip1/WAF1}$ pathway. (A) IMR-90 cells (PDL 32) were transfected with control or PLD2 siRNA. 5 h after transfection, the medium was changed and the cells were grown for 3 days. Cells were fixed and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside, after which the percentage of positively stained cells was measured (upper panel). Data are shown as the mean ± S.E.M. $^{**}P < 0.01$. Representative images were obtained at 20× magnification (bottom panels). (B) PLD2 siRNA-treated IMR-90 cells were lysed, electrophoresed on a 10% (w/v) SDS–polyacrylamide gel, and visualized by Western blotting with the appropriate antibodies. β-actin served as the loading control. Values below each band represent the mean fold differences ($n = 3$) in expression levels compared to the PLD2 siRNA negative control, which was assigned a value of 100. (C) Wild-type (WT), p21$^{-/-}$, and p53$^{-/-}$ HCT116 cells were treated with control or PLD2 siRNA. 5 h after transfection, the medium was changed and the cells were grown for 3 days. Cells were fixed and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside, after which the percentage of positively stained cells was measured (upper panel). Data are shown as the mean ± S.E.M. $^{**}P < 0.01$. Representative images were obtained at 20× magnification (bottom panels). (D) siRNA-treated HCT116 cells were lysed, electrophoresed on a 10% (w/v) SDS–polyacrylamide gel, and visualized by Western blotting with the appropriate antibodies.

^{WAF1} was upregulated only in the wild-type HCT116 cells treated with PLD2 siRNA (Fig. 2D). Taken together, these results indicate that the activation of p53–p21$^{Cip1/WAF1}$ pathway acts as a major mediator of PLD2 knockdown-mediated senescence.

3.3. PLD2 downregulation increases the intracellular ROS in a NADPH oxidase (NOX)-dependent manner

Since ROS stabilizes p53 [20], we examined whether ROS are generated in PLD2 knockdown-induced senescent cells by incubating IMR-90 and HCT116 cells with CM-H_2DCFDA or DHE. CM-H_2 DCFDA is hydrolyzed to DCFH, which is then oxidized by hydrogen peroxide to yield the fluorescent DCF [21]. DHE, which is relatively specific for the superoxide anion ($O_2^{-}\cdot$), is oxidized to the fluorescent ethidium (ETH) [22]. As shown in Fig. 3A, PLD2 siRNA significantly increased the production of hydrogen peroxide and superoxide anion in proliferating IMR-90 (PDL 32) as well as HCT116 cells, as indicated by the rightward shift in DCF and ETH fluorescence using flow cytometry. Incubation with N-acetyl-L-cysteine (NAC), which is a commonly used scavenger of ROS, appeared to inhibit the fluorescent signal in the PLD2 siRNA-treated cells. NOX produces superoxide anion using NADPH as the electron donor [23]. To confirm the involvement of superoxide anion in the cellular responses subsequent to PLD2 downregulation, proliferating IMR-90 (PDL 32) and HCT116 cells were incubated with the NOX inhibitor, apocynin. The presence of apocynin significantly suppressed PLD2 knockdown-induced ROS generation (Fig. 3B). Since p22^{phox} is a common integral component of the electron-transport machinery for NOX family enzymes [24], we knocked down p22^{phox} using siRNA in PLD2-inhibited cells. Transfection of p22^{phox} siRNA markedly decreased the PLD2 knockdown-mediated ROS generation (Fig. 3B). When cells were treated with apocynin or p22^{phox} siRNA alone as a negative control, ROS production decreased in the cells (Supplementary Fig. 1). To investigate whether PLD2 inhibition affects the mitochondrial superoxide generation, we stained proliferating IMR-90 (PDL 32) and HCT116 cells with MitoSOX Red, which selectively detects superoxide in the mitochondria of live cells [25]. As shown in Fig. 3C, transfection with PLD2 siRNA did not induce MitoSOX fluorescence. Next, we examined whether NAC and apocynin could rescue the cellular senescence induced by PLD2 downregulation. While treatment with PLD2 siRNA increased the SA-β-gal activity in IMR-90 (PDL 32) and HCT116 cells, co-treatment with NAC or apocynin resulted in a decrease in this activity (Fig. 3D). Similarly, co-treatment with NAC or apocynin caused cells to exhibit an apparently lower expression of p53 and p21$^{Cip1/WAF1}$ proteins compared with that in cells treated with PLD2 siRNA (Fig. 3E). Taken together, these results indicate that the superoxide anion generated by NOX is involved in the induction of senescence by PLD2 downregulation.

3.4. CK2α overexpression suppresses PLD2 knockdown-mediated senescence

We previously showed that the ROS–p53–p21$^{Cip1/WAF1}$ pathway acts as an important mediator of CK2 inhibition-induced cellular senescence [26–28]. Here, we demonstrate that this pathway is also necessary for PLD2 knockdown-mediated senescence. Thus, we examined whether CK2 was involved in the senescence induced

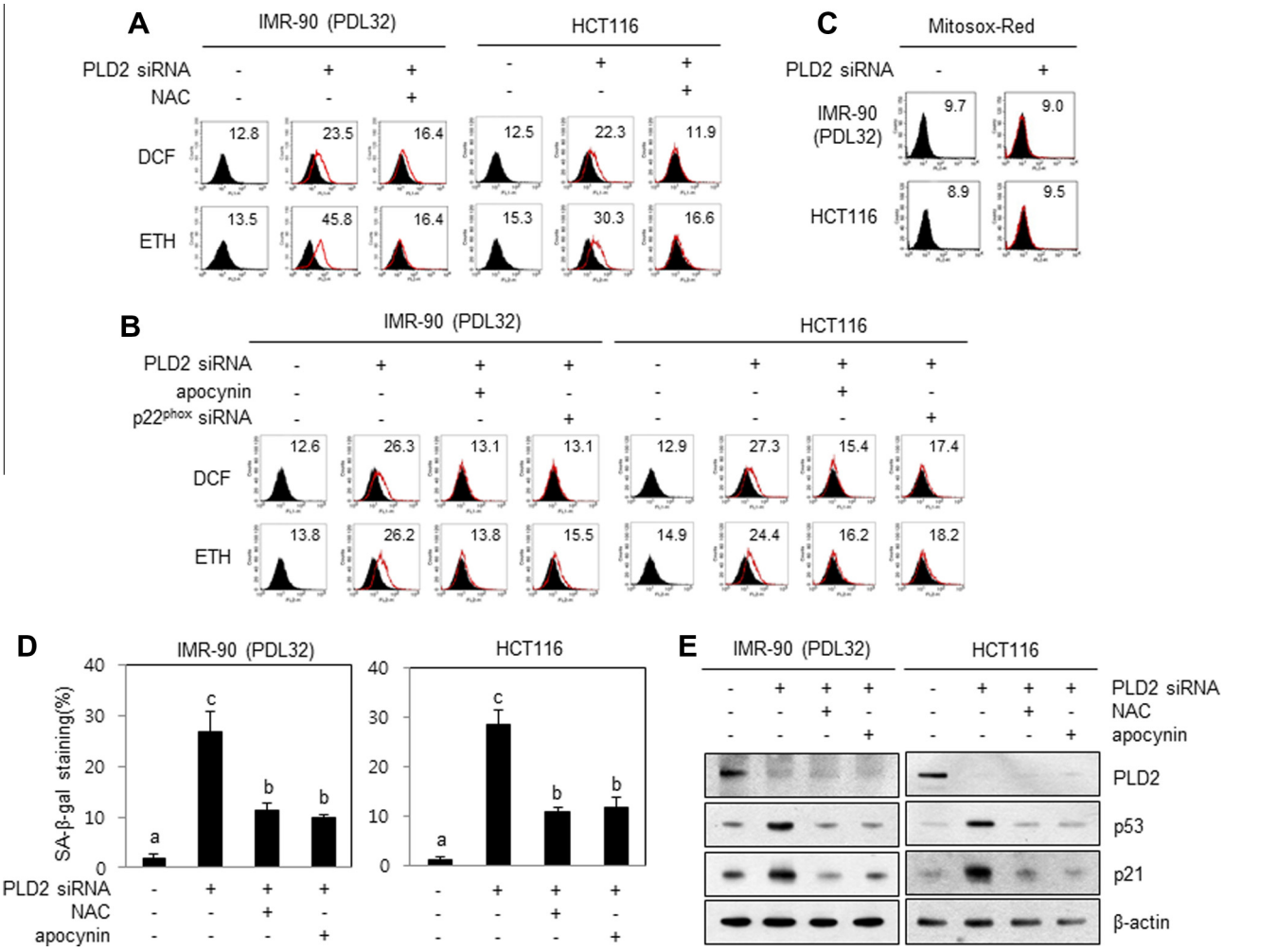

Fig. 3. PLD2 downregulation produces ROS via a NOX-dependent pathway in cells. (A and B) IMR-90 (PDL 32) and HCT116 cells were transfected with PLD2 siRNA grown for 2 days in the presence or absence of 10 mM NAC (A) and 10 μM apocynin or $p22^{phox}$ siRNA (B). The cells were then incubated with 5 μM CM-H_2DCFDA (upper panels) or DHE (bottom panels) as described in Section 2. The fluorescence intensity was determined by flow cytometry analysis. The mean fluorescence intensity is shown in the upper-right corners of the histograms. (C) IMR-90 (PDL 32) and HCT116 cells were transfected with PLD2 siRNA and grown for 4 days. The cells were then incubated with 5 μM MitoSOX Red as described in Section 2 and the fluorescence intensity was determined by flow cytometry analysis. (D and E) IMR-90 (PDL 32) and HCT116 cells were transfected with PLD2 siRNA in the presence or absence of 10 mM NAC or 10 μM apocynin and grown for 2 days. (D) Cells were fixed and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside as described in Section 2. The percentage of positively stained cells was measured. Values indicate mean ± S.E. Bars that do not share a common letter (a, b, c) are significantly different among the groups at $P < 0.05$. (E) Cells were lysed, subjected to electrophoresis on a 10% (w/v) SDS–polyacrylamide gel, and visualized by Western blotting with the appropriate antibodies.

by PLD2 downregulation. To induce senescence, proliferating IMR-90 (PDL 32) and HCT116 cells were transfected with PLD2 siRNA, and a significant increase in the SA-β-gal activity was found in response to PLD2 knock-down, whereas co-transfection of cells with CK2α resulted in a significant decrease in the level of SA-β-gal staining (Fig. 4A). Similarly, co-treatment of cells with CK2α evidently suppressed the upregulation of p53 and $p21^{Cip1/WAF1}$ (Fig. 4B) as well as the ROS generation (Fig. 4C) induced by PLD2 downregulation. Taken together, these data demonstrate that elevated CK2α expression can repress senescence induced by PLD2 downregulation.

3.5. Elevated PLD2 expression suppresses CK2 inhibition-mediated senescence through increased CK2 activity

We examined whether PLD2 modulates the senescence induced by CK2 inhibition. IMR-90 (PDL 32) and HCT116 cells were transfected with CK2α siRNA to induce senescence and then stained for SA-β-gal activity. Consistent with previous reports [26–28], we observed a significant increase in SA-β-gal activity in response to CK2α knockdown. However, co-transfection of cells with wild-type PLD2 resulted in a significant decrease in the level of SA-β-gal staining. In contrast, overexpression of the catalytically inactive mutant of PLD2 (K758R) did not affect SA-β-gal activity in cells (Fig. 5A). To further characterize the relationship between PLD activity and suppression of CK2 inhibition-induced senescence, PLD activity was assessed. Fig. 5B shows that CK2α knockdown resulted in a decrease in PLD activity that could be recovered by wild-type PLD2. The expression of p53 and $p21^{Cip1/WAF1}$ was upregulated in cells treated with CK2α siRNA, whereas co-treatment with the wild-type PLD2, but not the K758R mutant, reduced the expression of both p53 and $p21^{Cip1/WAF1}$ (Fig. 5C). Co-treatment with wild-type PLD2, but not the K758R mutant, also inhibited ROS generation induced by CK2α knockdown (Fig. 5D). Taken together, these data demonstrate that elevated PLD2 expression can repress senescence induced by CK2 inhibition, and that the catalytic activity of PLD2 is necessary for this event.

Previously, it has been demonstrated that PLD is activated and phosphorylated by CK2 in U87 astroglioma cells [29]. Based on our observation that PLD2 can inhibit CK2 inhibition-mediated

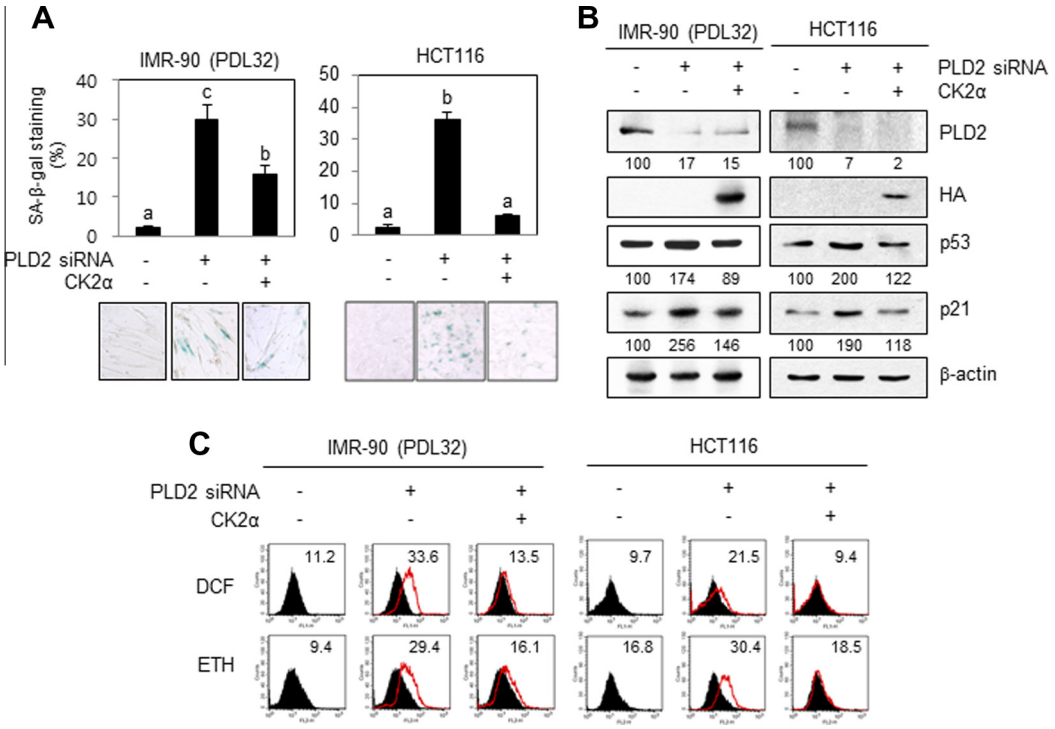

Fig. 4. CK2α overexpression suppresses PLD2 knockdown-mediated senescence in cells. IMR-90 (PDL 32) and HCT116 cells were transfected with PLD2 siRNA in the presence or absence of pcDNA3.1-CK2α and grown for 2 days. (A) Cells were fixed and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside, after which the percentage of positively stained cells was measured (upper panels). Data are shown as the mean ± S.E.M. Bars that do not share a common letter (a, b, c) are significantly different among the groups at $P < 0.05$. Representative images were obtained at 20× magnification (bottom panels). (B) Cells were lysed and subjected to electrophoresis on a 10% (w/v) SDS–polyacrylamide gel. Protein bands were then visualized by Western blotting with the appropriate antibodies. HA, hemagglutinin. (C) Cells were incubated with CM-H_2DCFDA or DHE as described in Section 2 and the fluorescence intensity was determined by flow cytometry analysis.

senescence, we tested whether PLD2 regulates the catalytic activity of CK2 in HCT116 and IMR-90 cells. The CK2 activity was assessed using [γ-^{32}P]ATP and the synthetic peptide substrate RRREEETEEE. As shown in Fig. 5E, the phosphotransferase activity of CK2 was approximately 25% higher in PLD2-overexpressing HCT116 and IMR-90 cells. In contrast, PLD2 knockdown reduced the activity of CK2 by 20% compared to the control cells. Nevertheless, the protein levels of CK2α did not change upon PLD2 overexpression or knockdown. Collectively, these data demonstrate that PLD2 can upregulate CK2 activity at the post-translational level.

4. Discussion

The results of the present study demonstrate that the expression of PLD1 and PLD2 decreases during replicative as well as premature senescence in IMR-90 cells. The apparent inverse relationship between the expression level of PLD1 and PLD2 and the growth of IMR-90 cells suggests that the decrease in PLD activity may be tightly coupled to the senescence process. In addition, our results demonstrate that the senescence-associated decrease in PLD expression is mainly regulated at the transcriptional level. Since PLD2 exhibited a higher basal level of cellular activity than PLD1 [1,2], we focused on the role of PLD2 in senescence in this study. The notion that downregulation of PLD may be linked to senescence is supported by the observation that exogenously added PLD2 siRNA could induce the senescent phenotype in IMR-90 cells. How can PLD downregulation induce cellular senescence? In the present study, we show that the expression of both p53 and p21$^{Cip1/WAF1}$ is upregulated in IMR-90 and wild-type HCT116 cells treated with PLD2 siRNA. In addition, senescence induced by PLD2 downregulation is strongly attenuated in p53$^{-/-}$ or p21$^{Cip1/WAF1-/-}$ HCT116 cells. Thus, this study demonstrates that the activation of p53–p21$^{Cip1/WAF1}$ pathway acts as a major mediator of PLD2 knockdown-induced senescence.

The tumor suppressor protein p53 is a potent transcription factor that plays a key role in cell cycle regulation. p53 is activated in response to a variety of cellular stress signals including DNA damage and triggers cell cycle arrest or apoptosis to prevent cells from undergoing transformation [20]. The present study indicates a possible mechanism by which PLD2 downregulation can stabilize and/or activate p53. First, PLD2 downregulation induced DCF and ETH signals, which were blocked by the NAC treatment, indicating the cellular production of hydrogen peroxide and superoxide anion. Second, apocynin and p22^{phox} siRNA almost completely abolished the PLD2 knockdown-induced increase in ROS. Finally, NAC and apocynin successfully rescued the p53 stabilization and senescence induced by PLD2 downregulation. Thus, these findings indicate that NOX-dependent superoxide anion production is a major upstream contributor of the PLD2 knockdown-mediated senescence signaling cascade.

CK2 is known to be a second messenger-independent serine/threonine kinase. The holoenzyme of CK2 is a heterotetramer composed of two catalytic (α) and two regulatory (β) subunits [30]. Dysregulated CK2 activity has been implicated in the development and progression of a variety of tumor or leukemic cells [31–33]. In addition, CK2 reportedly phosphorylates caspase substrates, preventing apoptosis [34]. These observations suggest that CK2 plays a pivotal role in diverse cellular processes such as cell growth, proliferation, and apoptosis. We have previously shown that CK2 inhibition induces cellular senescence via the ROS–p53–p21$^{Cip1/WAF1}$ pathway [26–28]. Our results signify that enhanced CK2 activity significantly suppresses PLD2 knockdown-mediated senescence and ROS production in IMR-90 and HCT116 cells. In addition, elevated PLD2 expression also suppresses senescence as well as ROS

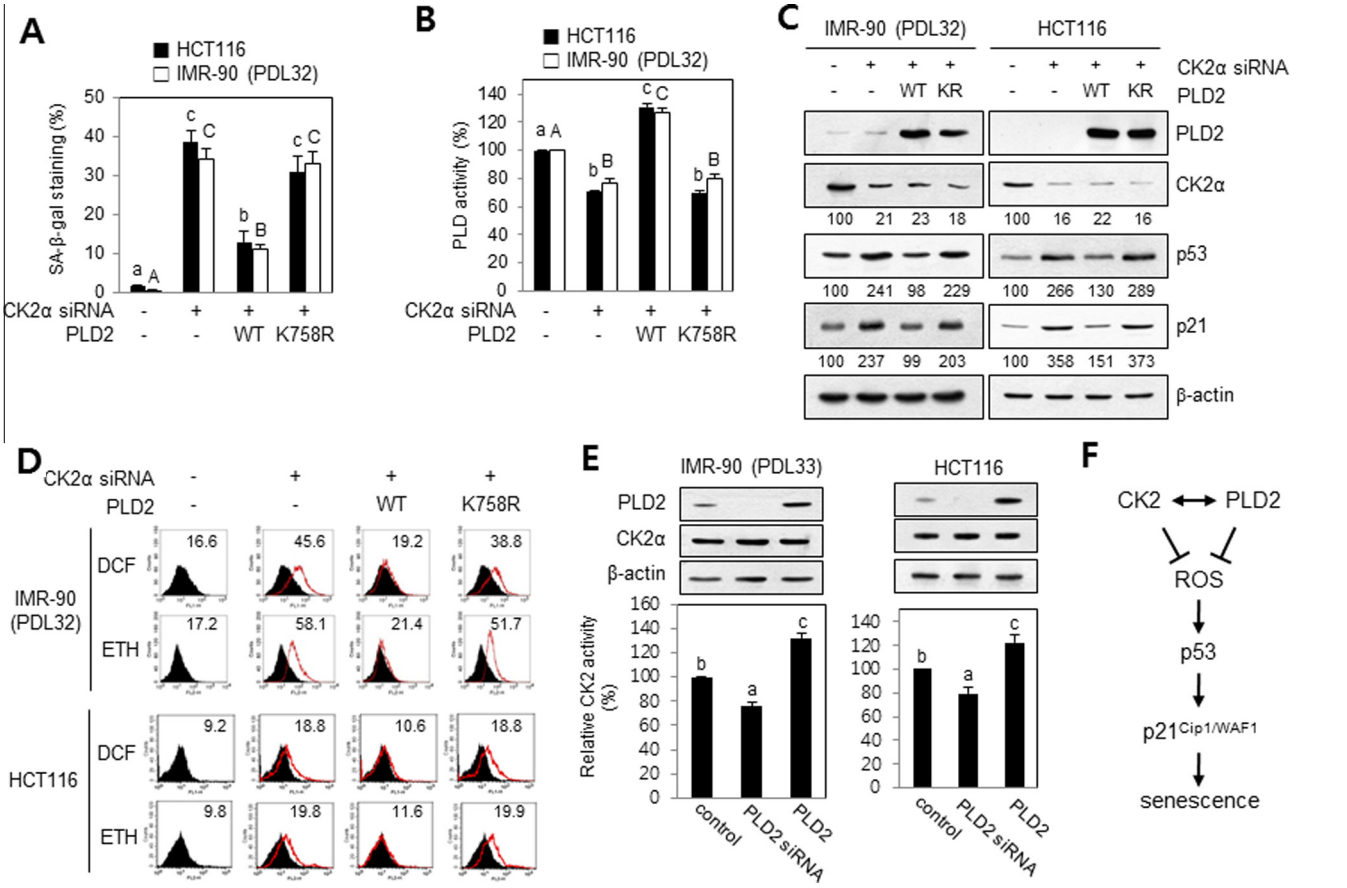

Fig. 5. Elevated PLD2 expression suppresses senescence through the increase of CK2 activity. (A–D) IMR-90 cells (PDL 32) and HCT116 cells were co-transfected with wild-type (WT) or mutant (K758R, KR) PLD2 in the presence of CK2α siRNA and grown for 2 days. (A) Cells were fixed and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside, and the percentage of positively stained cells was measured. (B) Cells were collected, and PLD activity was determined as described in the Section 2. (C) Cells were lysed, electrophoresed on a 10% (w/v) SDS–polyacrylamide gel, and visualized by Western blotting with the appropriate antibodies. (D) Cells were incubated with CM-H_2DCFDA (upper panels) or DHE (bottom panels) as described in Section 2 and the fluorescence intensity was determined by flow cytometry analysis. (E) IMR-90 (PDL 33) and HCT116 cells were transfected with PLD2 siRNA or pcDNA3.1-PLD2 and grown for 2 days. Cell lysates were visualized by Western blotting with the appropriate antibodies (upper panels) and assessed for CK2 activity (bottom panels). Data are shown as the mean ± S.E.M. Bars that do not share a common letter (a, b, c or A, B, C) are significantly different among the groups at $P < 0.05$. (F) Putative model illustrating PLD2 knockdown-mediated cellular senescence. PLD2 downregulation results in the activation of the p53–p21$^{Cip1/WAF1}$-dependent cellular senescence pathway through ROS generation, which is mediated via CK2 inhibition. CK2 stimulates PLD2 activity in human cells [29].

generation induced by CK2 inhibition. Furthermore, overexpression of PLD2 was found to stimulate the catalytic activity of CK2, whereas CK2 activity decreased in response to PLD2 knockdown. Collectively, the present study demonstrates that downregulation of PLD2 promotes senescence through a pathway identical to that of the CK2 inhibition-mediated senescence (Fig. 5F). In a previous study, CK2 was shown to phosphorylate and activate PLD2 in human cells [29]. PKC has been previously reported to enhance CK2 activity through CK2β phosphorylation in vitro [35]. Our recent data suggested that PLD2 stimulated PKC catalytic activity, which was responsive for CK2α phosphorylation and subsequent stimulation of CK2 activity. Furthermore, PKC-specific activator stimulated CK2 activity in cells, whereas PKC inhibitor suppressed the activity (Lee and Bae, manuscript in preparation).

Increasing evidence over the last few decades has implicated cellular senescence as an important anti-cancer defense mechanism. Thus, understanding the underlying mechanisms of premature senescence may contribute to the development of improved and effective cancer treatments. In summary, this study has demonstrated a new senescence signaling pathway that specifically involves PLD2. PLD2 downregulation decreases CK2 activity, leading to NOX-dependent ROS generation with subsequent activation of p53 and p21$^{Cip1/WAF1}$. These novel observations will provide a better understanding of cellular senescence as well as novel diagnostic and therapeutic options for the treatment of various tumors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.07.009.

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